GENETIC STRUCTURE OF *VACCINIUM PARVIFOLIUM* (ERICACEAE) IN NORTHERN CALIFORNIA REVEALS POTENTIAL SYSTEMATIC DISTINCTIONS

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ABSTRACT

Vaccinium parvifolium Sm. (Ericaceae) is an important understory shrub in conifer forests in western North America. Populations putatively classified as *V. parvifolium* in northern California display alternate berry morphology, consistent with a possible phenotypic diversification or cryptic speciation. Identification of cryptic species or subspecies would influence management guidelines given the limited range of some morphological variants. In order to inform management guidelines, two *Vaccinium* species were characterized via molecular genetic analyses. Plants of typical *V. parvifolium* morphology from the coastal areas of northwest California, western Oregon and Washington, atypical plants from Shasta County and the central Sierra Nevada, and one population of *V. deliciosum* Piper, a congener, were assessed at five nuclear microsatellite loci. Analyses of differentiation, admixture, and phylogenetic relationships indicated that populations displaying atypical morphology were more similar to *V. deliciosum* than to the typical *V. parvifolium*. Although additional data are required to determine whether these differences warrant taxonomic treatment within *Vaccinium*, management plans should consider three distinct gene pools among these populations.

Key Words: Ericaceae, management units, microsatellites, population genetics, Shasta County, taxonomy.

Members of the genus *Vaccinium* (Ericaceae) are ecologically important shrubs in the forests of the Pacific Coast of North America, where they may dominate understory vegetation especially in response to disturbance or thinning (Hanley 2005). *Vaccinium parvifolium* Sm., the red huckleberry, is a perennial understory shrub that occurs from Alaska south through British Columbia and into California. The stems of *V. parvifolium* reach 4 m in height and are sharply angled, giving shrubs a characteristic architecture, and the translucent red, edible berries are highly distinguishing (Wallace 1993, accessed in Rosatti 2003; Vander Kloet 2009). Plants are not rhizomatous but produce horizontal stems capable of rooting, providing a means of vegetative reproduction which can maintain genets (genetic individuals) over many years. The *V. parvifolium* flower color is described as pink, bronze or yellowish green, and they produce red berries up to 10 mm in diameter (Vander Kloet 2009). In addition to having edible fruit, *V. parvifolium* vegetation is highly palatable to deer (Vila et al. 2004). As an angiosperm shrub in conifer forests, *V. parvifolium* likely plays a critical role in species diversity and ecological systems (Wender et al. 2004).

Populations of putative *V. parvifolium* in two regions of California, Shasta County and the central Sierra Nevada, do not display the typical morphology (Fig. 1). Populations in the area around Shasta Lake, in Shasta County, California display white to pink urceolate flowers with glaucous berries. Plants grow in distinct clumps of or as individual ramets, typically 1 to 2 m in height, but some reach up to 3 m tall. Plants are erect and do not root along stems. The Shasta populations are found in conifer and hardwood-conifer forest, and chaparral habitats; typically in vegetation types classified as Douglas-fir, ponderosa pine, montane-hardwood-conifer, and mixed chaparral (Mayer and Laudenslayer 1988). Shasta County *Vaccinium* are associated with riparian areas, springs and seeps, and mesic forest environments. These sites are distinguished from the habitats of typical *V. parvifolium* by substantially higher summer temperatures and more extended summer drought. Further, the Shasta County populations are in areas characterized by acidic soil and/or water conditions. In
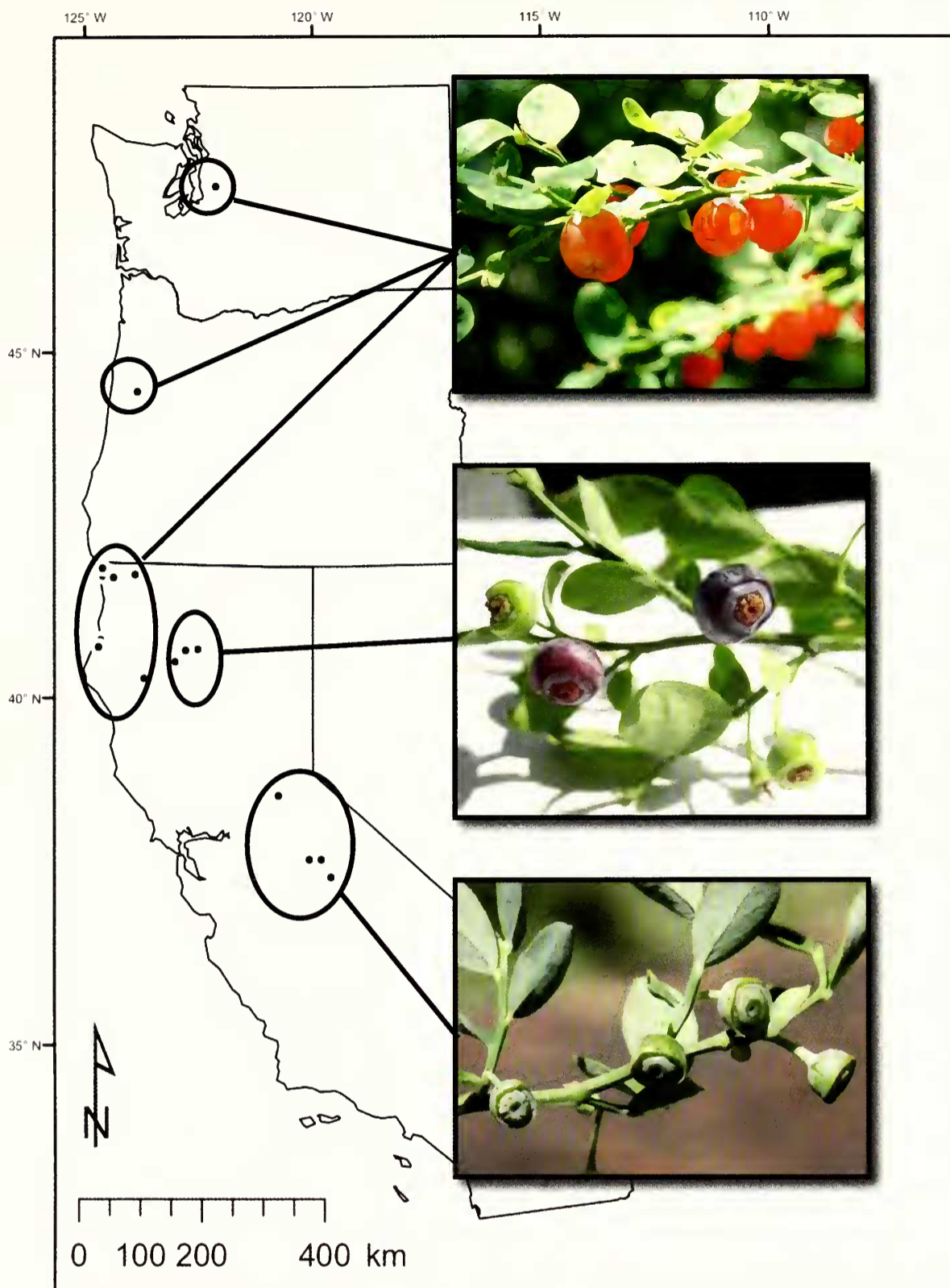
FIG. 1. Morphological variation among *Vaccinium parvifolium* populations sampled for genetic analyses. The typical red-berry morphology observed in the coastal areas (top), purple-colored berry observed in Shasta County (middle), and a distinctive calyx ring on berries that mature to a blue-black color in the central Sierra Nevada (bottom).

many situations the species grows immediately downstream of historic mines in the acidic soil and water. The species also regularly occurs in riparian areas with acid mine discharge water chemistry. These inland Shasta County populations are disjunct from the nearest known extant red huckleberry populations in the coastal region by approximately 60 km, with the Trinity Alps and other Klamath Ranges lying between them (J.K.N., personal observation and review of herbarium records).

Vaccinium populations in the central Sierra Nevada display white urceolate flowers with blue-black berries that have a calyx ring, and plants are decumbent and capable of rooting along branches

(Fig. 1). The Sierra Nevada plants grow along drainages in *Pseudotsuga menziesii-Abies concolor-Calocedrus decurrens* and in *Abies concolor-Pinus lambertiana-Calocedrus decurrens/Cornus nuttallii/Corylus cornuta* var. *californica* forest associations, intermediate in moisture level between the Shasta County form and the typical form. The atypical populations in the Sierra Nevada are more than 250 km from the Shasta County populations. All specimens observed from the Shasta County and Sierra Nevada regions display the atypical forms (A. E. L. Colwell, National Park Service, personal communication).

The taxonomy of the *Vaccinium* genus is of ongoing study due to the wide range and high

levels of phenotypic variation observed within species (Vander Kloet and Dickinson 1999; Powell and Kron 2002). Members of the *Vaccinium* genus are found in the Northern Hemisphere circling the Pacific Rim from Japan to Mexico, with a center of species and habitat diversity ranging from Alaska to California. No clear geographical pattern was distinguished in the grouping of *Vaccinium* species, and the full phylogeny of the clade has not been resolved, requiring greater sampling to better represent the approximately 20 species of *Vaccinium* in North America (Kron et al. 2002; Powell and Kron 2002).

Given the ecological importance of *Vaccinium* shrubs in conifer ecosystems, the unresolved nature of the systematic classifications within the genus, and the morphological variation observed in northern California, additional study of *V. parvifolium* was warranted. Identification of landscape patterns of genetic differentiation or cryptic species can influence estimates of species diversity and local adaptation, and inform conservation plans. Although most studies using molecular markers to identify cryptic species have focused on animals (Bickford et al. 2006), the use of molecular markers in plant species is well established in studies of population genetics (Hamrick and Godt 1996) and may aid efforts to resolve questions of speciation and divergence (Lawton-Rauh 2008).

Here, we used molecular data to investigate the genetic relationship of *Vaccinium* populations primarily from northern and central California. *Vaccinium parvifolium* populations from northern California and western Oregon and Washington (the typical red huckleberry) were compared to collections displaying atypical morphologies from Shasta County and the central Sierra Nevada. In addition, one population of the congener *Vaccinium deliciosum* Piper was included from Shasta County. *Vaccinium deliciosum*, the Cascade bilberry, is typically shorter than *V. parvifolium* but is rhizomatous and capable of forming dense clonal mats (Wallace 1993; Vander Kloet 2009). Vaccinium *deliciosum* flowers range from creamy pink to red, and fruit are typically blue but may be black or maroon or sometimes red. These species are closely related, with *V. parvifolium* and its sister taxa *V. ovalifolium* Sm. composing the ancestral clade to *V. deliciosum* (Powell and Kron 2002). Specifically, our objectives were to quantify the level of genetic differentiation among populations and geographic regions, to determine whether the populations from Shasta County and the Sierra Nevada are likely to be morphological variants of *V. parvifolium* or may be taxonomically distinct, and to provide data to inform ongoing conservation efforts in Shasta County.

Materials and Methods

Study Species and Collections

In order to investigate the genetic structure of *Vaccinium parvifolium*, five categories of populations were sampled for genetic analyses (Table 1, Fig. 2). We sampled populations of atypical morphology from regions lacking the typical red-berried *V. parvifolium*. These categories represent distinct geographic regions with limited *V. parvifolium* distribution between. Five populations displaying the unusual berry color and morphology were sampled from the Shasta County area (category 1). Ten populations displaying the typical *V. parvifolium* berry color and morphology were sampled: eight from northwestern California, relatively proximate to Shasta County (category 2), and two from western Oregon and Washington (category 3). Four populations of dark-fruited *Vaccinium* were sampled from the central Sierra Nevada (category 4). Finally, one population of *V. deliciosum* from western Shasta County was sampled for interspecific comparison (category 5). Voucher specimens were collected from each population in order to verify and document species identification.

Collections were made over three growing seasons (2004, 2005 and 2009). Up to 20 samples were collected from each population. Population locations were recorded in the field using GPS or topographic maps. Samples from the coastal and Shasta County regions (categories 1 and 2) were collected from distinct shrubs randomly selected from throughout the population. The Sierra Nevada samples (category 4) were spaced to minimize duplicate collection of clones. Leaf material was collected in the field and stored on wet ice and/or refrigerated until shipped to the USDA Forest Service National Forest Genetics Lab (NFGEL).

DNA Isolation and Analysis

Total genomic DNA was isolated from all samples using the DNEasy-96 plant kit (Qiagen, Valencia, CA) following the liquid nitrogen procedure as described in the manual. DNA concentrations were quantified using a Gemini XPS Microplate Spectrofluorometer (Molecular Devices, Sunnyvale, CA) using PicoGreen dsDNA Reagent (Invitrogen, Carlsbad, CA). Samples were assayed for five microsatellite loci originally described in *Vaccinium corymbosum* L.: CA23F, CA421, CA787F, VCC1_I2, and VCC1_J9 (Boches et al. 2005). Amplification of the five loci took place in two multiplex reactions: CA421 with CA787F in one reaction, and CA23F, VCC1_I2, and VCC1_J9 in a second reaction. Amplification conditions included 10 ng of template DNA, 2 μM of each primer, and 1× of Multiplex master mix. Both amplifications took place following the

Table 1. Location and Classification of 20 Populations of *Vaccinium* spp. Sampled for DNA Analyses. Populations were grouped into four categories according to morphology, geography, and species designation. [a]Population 2SR was collected from three sites forming one distinct population. The three collections were combined for all statistical analyses.

Abbrev.	Population	County, State	n	Latitude/ Longitude	Elev. (ft)
Atypical *Vaccinium* in Shasta County					
1BH	Bully Hill, Shasta Lake	Shasta, CA	20	40.7882/−122.2087	1100
1FLM	Friday-Lowden Mine, Shoemaker Gulch	Shasta, CA	26	40.7534/−122.4599	2400
1LB	Little Backbone Creek, Shasta Lake	Shasta, CA	20	40.7611/−122.4376	1100
1SQC	(Little) Squaw Creek, Shasta Lake	Shasta, CA	20	40.7397/−122.4689	1100
1ULB	Upper Little Backbone Creek, Shasta Lake	Shasta, CA	20	40.77/−122.4444	2000
Typical *V. parvifolium*, Pacific coast of California					
2ACF	Arcata Community Forest	Humboldt, CA	33	40.8772/−124.0799	41
2HC	Happy Camp, Doolittle Creek Drainage, Forest Road 17N62	Siskiyou, CA	27	41.84/−123.45	3280
2HD	High Divide, Hiouchi, CA	Del Norte, CA	2	41.8029/−124.0625	42
2JS	Jedediah Smith State Park, Redwood National Park, Redwood State Park	Del Norte, CA	15	41.8083/−124.0894	42
2LD	Low Divide, Hiouchi, CA	Del Norte, CA	2	40.7798/−124.1144	41
2LR	Lentell Road, Eureka, CA	Humboldt, CA	2	41.9146/−124.1144	42
2SFM	South Fork Mountain	Trinity, CA	20	40.3474/−123.221	4700
2SR[a]	Six Rivers National Forest	Del Norte, CA	4	41.7683/−123.8852	3090
2SR[a]	Six Rivers National Forest, Camp Six	Del Norte, CA	4	41.8268/−123.8729	3600
2SR[a]	Six Rivers National Forest, Gordon Mtn.	Del Norte, CA	4	41.7884/−123.8713	3824
Typical *V. parvifolium*, western Oregon and Washington					
3I	Issaquah, Christmas Lake	King, WA	1	47.432/−121.760	4250
3MP	Mary's Peak Recreation Area	Benton, OR	23	44.4957/−123.5436	2575
Atypical *Vaccinium* in the central Sierra Nevada					
4BC	Big Creek, Yosemite National Park	Mariposa, CA	22	37.5083/−119.6604	4630
4CC	Clear Creek Road	El Dorado, CA	20	38.6901/−120.6361	2600
4GH	Greeley Hill	Tuolumne, CA	19	37.7683/−120.0625	3281
4MC	Moss Creek, Yosemite National Park	Tuolumne, CA	20	37.7638/−119.8332	5972
V. deliciosum					
5VDE	Shasta Bally, Whiskeytown National Park	Shasta, CA	20	40.6011/−122.6437	5900

program provided with the Multiplex PCR Kit (Qiagen). Forward primers for all loci were labeled with a fluorescent tag for visualization on an ABI3130xl capillary electrophoresis system (Applied Biosystems, Carlsbad, CA): CA23F and CA787F labeled with HEX, CA421 and VCC1_I2 with 6FAM, and VCC1_J9 with NED. Electrophoresis was conducted using a 1:100 dilution of amplification products.

Data Analysis

In order to quantify the extent of vegetative reproduction in *Vaccinium*, multiple ramets of the same genet were identified as matching multilocus genotypes using the Multilocus Matches tool in GenAlEx v. 6 (Peakall and Smouse 2006). Missing data were considered sufficient to identify a mismatch. The genet diversity of each population was quantified as the number of unique genotypes observed per sample (G/N). Differences in genet diversity among geographic regions (coastal, Shasta, and Sierra) were determined using univariate ANOVA as implemented in SPSS v. 17.0 (IBM SPSS, Chicago, IL). The probability of identity function was then used to determine the expected number of unrelated genets with the same multilocus genotype in each
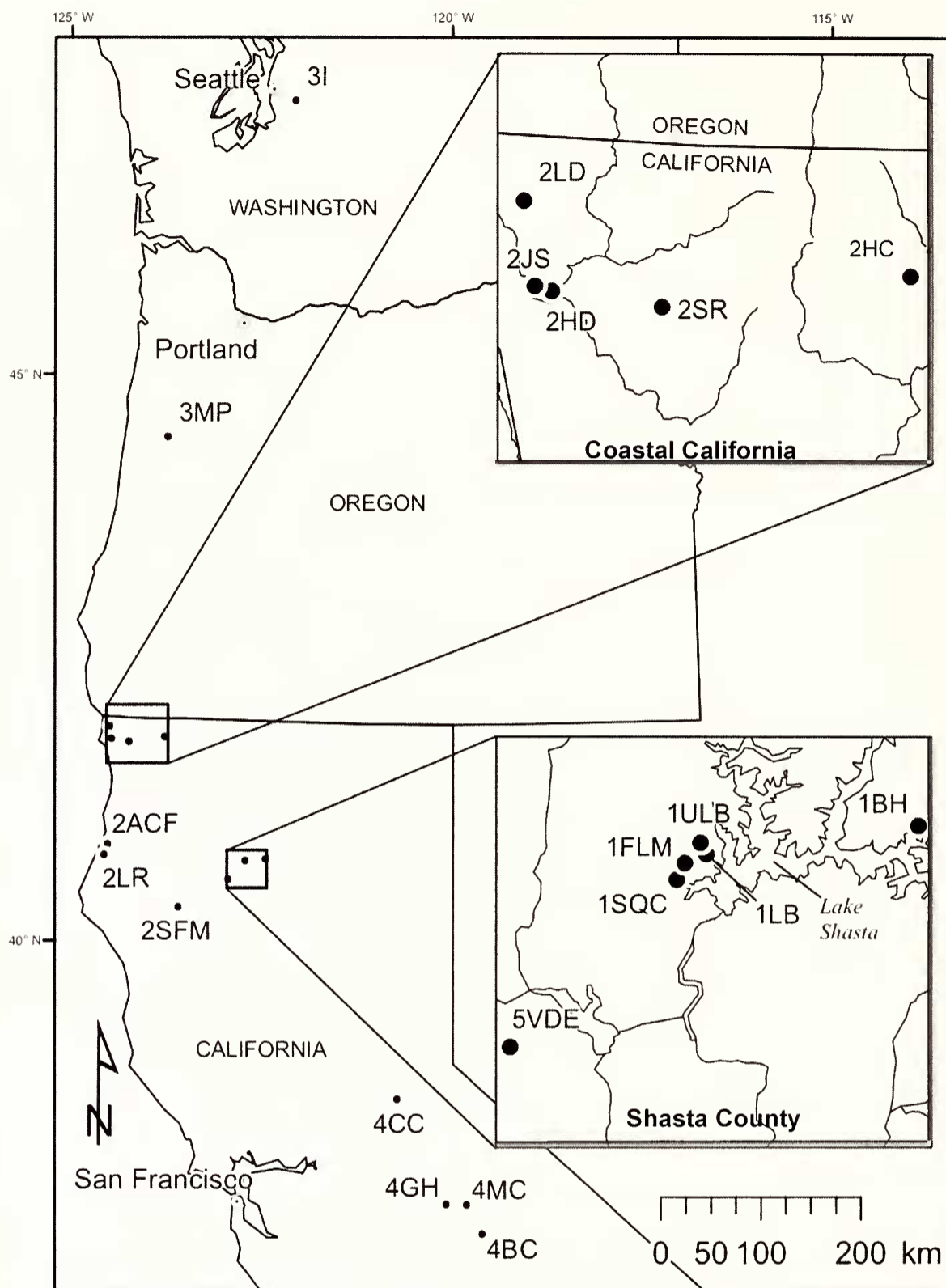
Fig. 2. *Vaccinium* populations were sampled in western Oregon and western Washington, coastal California, Shasta County, and the Sierra Nevada. The Shasta County populations included one population of *V. deliciosum* (5VDE). Population abbreviations follow Table 1.

population. When multiple ramets were identified in a population, the data set was reduced to unique genets (one stem per genotype) and assessed for basic measures of allelic diversity, including the percent polymorphic loci (P), mean alleles per locus (A), effective alleles per locus (A_e) (which standardizes for varying sample sizes), observed (H_o) and expected heterozygosity (H_e), and the within-population fixation index (F). All measures were estimated using GenAlEx v. 6 (Peakall and Smouse 2006). When sufficient samples were present (10 genets), populations were tested for an excess of heterozygotes relative to mutation-drift equilibrium using the Wilcoxon sign-rank test under a two-phase mutation model

as implemented by Bottleneck (Cornuet and Luikart 1996).

When applying microsatellite markers to different species, mutation of the PCR primer site may produce null alleles that do not produce visible products. These null alleles may inflate certain genetic measures such as the fixation index (or estimated inbreeding) and decrease other measures (such as heterozygosity). As the markers used in this study were not designed specifically for *V. parvifolium* or *V. deliciosum*, null alleles must be taken into account for some analyses. In order to detect null alleles, those populations with sufficient sample sizes (unique genotypes) were assessed using Micro-Checker v2.2.3 (van Oosterhout

et al. 2004). When null alleles were detected, the adjusted allele frequencies and genotypes (randomized within the population) were calculated using the method of Brookfield (1996) where no null-null homozygotes are expected.

Both raw data and the null-adjusted genotypes were then used to estimate measures of population differentiation using analysis of molecular variance (AMOVA) over two models. The first model assumed no hierarchical structure and estimated the differentiation of all populations relative to the total. The second model tested for hierarchical structure among geographic regions of populations, and estimated differentiation among populations within each region, and among regions relative to the total collection. The hierarchical model grouped populations geographically and taxonomically into four groups: the atypical *Vaccinium* from Shasta County (category 1), typical *V. parvifolium* from the Pacific coast and western Oregon (categories 2 and 3), the atypical *Vaccinium* from the central Sierra Nevada (category 4), and *V. deliciosum* (category 5). The two populations with a single genet sampled (2LD and 3I, Table 1) were dropped from the AMOVA. Significance of differentiation was determined by 999 bootstrap replicates, as implemented in GenAlEx v. 6 (Peakall and Smouse 2006).

Allele frequencies (both raw and adjusted for null alleles) were used to estimate Nei's (1972) genetic distance for all pairs of populations. The distance matrices were then used to test if gene flow decreases as a function of geographic distance (isolation by distance, IBD). The latitude and longitude of each population was used to calculate the linear distance between sites in km. The correlation between the genetic and geographic distance matrices was assessed using the Mantel Test procedure as implemented by GenAlEx v. 6 (Peakall and Smouse 2006). Significance of the correlation was assessed from 999 permutations of the dependent (genetic) matrix relative to the independent (geographic) matrix. The distance matrices (raw and null-corrected) were also used to build consensus population phenograms using Neighbor-Joining methods, with significant branches identified from 100 bootstrap replicates following the extended majority rule option. Phenograms were built using the applications in PHYLIP (Felsenstein 2005).

Raw multilocus genotypes were treated to two unsupervised multivariate analyses to identify genetic structure not predicted a priori. First, the genetic distance matrix for all pairs of populations was treated to a Principle Coordinate Analysis (PCoA) as implemented by GenAlEx (Peakall and Smouse 2006). The analysis was conducted on the standardized covariance matrix, with unique genotypes represented for each population.

Second, raw genotypes were assigned to anonymous genetic clusters using Markov Chain Monte Carlo simulations and Bayesian likelihood methods as implemented in the program Structure v. 2.3.3 (Pritchard et al. 2000). This analysis is a robust method to identify clusters of similar multilocus genotypes even in the presence of admixture (Pritchard et al. 2000; Falush et al. 2003). To account for a low frequency of null alleles, the analysis was conducted with the assumption of unreported recessive alleles, allowing for homozygous genotypes to be considered as putative heterozygotes for an undetected (null) allele (Falush et al. 2007; Pritchard et al. 2007). For these data, simulations were undertaken to test for the most likely number of clusters for the set $K = \{1:10\}$, over 100,000 replications following a burn-in period of 50,000 replications, with no prior population information provided. The parameters were set to assume allele frequencies were uncorrelated among populations, which is feasible given the taxonomic and geographic scale of this study. For example, at least two distinct species have been sampled, and many populations are separated by greater distances than the typical pollinator, bees, are expected to travel regularly. Using the uncorrelated frequencies may merge similar populations and deflate estimates of K, while correlated frequencies may inflate K (Pritchard et al. 2000). The parameter set did allow for admixture within populations and individuals, which can account for migration or hybridization events. Calculations are based on the mean likelihood of five simulations for each value of K. The number of genetic clusters (K) was inferred from the test statistic dK following Pritchard et al. (2000), and individual assignments were assessed visually for the simulation with the greatest likelihood value. In addition, the genetic relationship of the inferred genetic clusters was visualized as a population phenogram using Neighbor-Joining methods (Saitou and Nei 1987) visualized using the DrawTree application from PHYLIP (Felsenstein 2005).

Results

Matching multilocus genotypes were identified in 12 populations (including *V. deliciosum*), indicating *Vaccinium* reproduces vegetatively with some frequency. The majority of individuals sampled had a unique multilocus genotype (mean number of samples per genotype = 1.24, $s_{\bar{x}}$ = 0.05), with a maximum of 11 stems found to share one genotype (population 4BC, where all stems were in a single, large patch). Resulting measures of genet diversity (G/N) were high (mean = 0.81, $s_{\bar{x}}$ = 0.05), with values ranging from 0.27 to 1.0 (Table 2). An analysis of variance indicated the levels of G/N to vary

TABLE 2. GENOTYPIC AND ALLELIC DIVERSITY AMONG POPULATIONS OF *VACCINIUM* SPP. ASSESSED AT FIVE MICROSATELLITE LOCI. G = Number of unique multilocus genotypes; n = Sample size; P = Percent polymorphic loci; A = Mean alleles per locus; A_e = Effective alleles per locus; H_o = Observed heterozygosity; H_e = Expected heterozygosity; F = Fixation index; deviation from Hardy-Weinberg equilibrium may be affected by the presence of null alleles.

Pop.	G	n	G/N	P	A	A_e	H_o	H_e	F
1BH	12	20	0.60	80	3.4	2.6	0.483	0.438	0.082
1FLM	22	26	0.85	60	4.0	2.7	0.318	0.414	0.271
1LB	20	20	1.00	80	5.2	3.2	0.393	0.399	−0.003
1SQC	10	20	0.50	80	3.8	2.6	0.360	0.451	0.160
1ULB	20	20	1.00	80	4.8	3.2	0.370	0.367	−0.032
2ACF	33	33	1.00	80	10.0	5.2	0.473	0.603	0.227
2HC	26	27	0.96	100	8.6	5.3	0.496	0.579	0.261
2HD	2	2	1.00	50	1.8	1.7	0.600	0.325	−0.867
2JS	15	15	1.00	80	8.0	4.4	0.554	0.604	0.073
2LD	1	2	0.50	50	1.6	1.6	0.600	0.300	−1.000
2LR	2	2	1.00	80	2.2	2.1	0.500	0.425	−0.167
2SFM	20	20	1.00	100	10.4	6.2	0.536	0.633	0.146
2SR	12	12	1.00	80	7.2	4.5	0.570	0.603	0.082
3I	1	1	1.00	60	8.2	6.0	0.583	0.609	0.001
3MP	22	23	0.96	80	1.6	1.6	0.600	0.300	−1.000
4BC	6	22	0.27	60	2.0	1.6	0.467	0.281	−0.593
4CC	16	20	0.80	60	4.0	2.5	0.263	0.325	0.132
4GH	13	19	0.68	60	3.8	2.8	0.338	0.389	0.291
4MC	13	20	0.65	100	4.4	2.3	0.277	0.381	0.378
5VDE	13	20	0.65	60	4.6	3.4	0.491	0.478	−0.020
Mean	13.95	17.2	0.82	75	4.98	3.28	0.464	0.445	−0.080

among geographic groups (Shasta County, coastal area, or Sierra Nevada) of putative *V. parvifolium* (*V. deliciosum* excluded) ($F_{2,16}$ = 4.66, P = 0.026).

The estimated probability that two unrelated genets displayed the same multilocus genotype (probability of identity, PI) varied among collections. The greatest PI occurred in populations with small sample sizes (2LD, PI = 0.05; 2HD, PI = 0.03) or in the population with the highest frequency of clonality resulting in a low effective sample size (4BC, PI = 0.05). The remaining populations displayed lower PI values, ranging from 2.5 × 10^{-6} to 0.008. When PI values were used to estimate the predicted number of unrelated genets with the same genotype in each population, based on sample sizes, only one population (4BC) was expected to have one duplicate genet (1.004), with all other values less than 0.2. These values indicate that while the five microsatellite loci did not provide universally low PI values in each population, the sampling strategy was sufficient to conclude that duplicate genotypes are most likely ramets of one genet and not a consequence of low statistical power.

All loci displayed allelic variation, although some populations were fixed for single variants (Table 2, Supplemental Material). Allelic diversity varied among populations, with the average number of alleles ranging from 1.6 to 10.4 per population. This range is likely influenced by differences in sample sizes, as a smaller range was estimated for the effective alleles per population,

which accounts for differences in sample number (mean 3.3 alleles, range 1.6 to 6.2). Moderate levels of heterozygosity were observed (mean H_o = 0.464), with levels of fixation ranging from −1.0 to 0.378 (mean −0.08).

No evidence was found of genetic bottlenecks. All tests for heterozygosity excess relative to mutation-drift equilibrium were non-significant.

Tests of per-locus excess homozygosity revealed the occurrence of null alleles to be variable among loci and populations. Four populations contained an insufficient number of unique genotypes for the Micro-Checker (van Oosterhout et al. 2004) analysis: 2HD, 2LD, 2LR, and 3I. Evidence of null alleles was found in four of the five loci. Null alleles were detected at locus CA421 in populations 2ACF, 2JS, 3MP, and 4MC; at locus CA787F in populations 2ACF, 4GH, at locus VCC1_I2 in populations 1FLM and 5VDE, and at locus VCC1_J9 in populations 2HC, 2SR, 3MP, and 4CC (Supplemental Material). The frequency of the detected null ranged from low (0.071) to moderate (0.262), indicating that null alleles may have inflated fixation indices (indicating a deficit of heterozygotes) in these collections. Genotypes adjusted for null alleles at each affected locus were randomized within each population and used for population-level analyses of differentiation and genetic distance.

Analysis of molecular variance over the null-corrected data revealed significant differentiation among populations and categories of populations in this collection of *Vaccinium*. The one-level

model estimated 58% of the variance to be contained within populations and 42% of the genetic variance to be partitioned among populations (P < 0.001). Significant differentiation was observed in the two-level hierarchical model based on geographic location: 51% of the variance was contained within populations, 10% of the variance was distributed among populations within a region, and 39% among geographic regions (P < 0.001). Estimates of differentiation from the raw genotypic data were congruent to those from null-corrected data (data not reported), indicating the occurrence of null alleles in the data set was insufficient to bias the detection of differentiation among these populations.

Levels of genetic distance calculated from the corrected genotypes varied among pairs of populations (Table 3). Genetic distances were greater between populations in different geographical regions, and smaller among populations within geographic areas. This pattern was confirmed by tests for isolation by distance (IBD), which identified a significant positive correlation between genetic and geographic distance among all pairs of populations for both raw ($R_{XY} = 0.38$, P < 0.001) and null-corrected data ($R_{XY} = 0.39$, P = 0.003). This pattern is consistent with the AMOVA analyses indicating greater differences among regions than among populations within a region.

Population phenograms built from the raw or null-corrected data were mostly congruent in their topology but neither was statistically significant (all branches were observed in less than 60% of bootstrap replicates). In both phenograms, populations grouped by geography, with the coastal populations (categories 2 and 3) forming one clade, the Sierra Nevada populations (category 4) a second clade, and the Shasta County populations (category 1) a third clade (Fig. 3). Potentially the most informative difference between the raw and null-corrected phenograms involved the placement of the single population of *V. deliciosum* (5VDE). In the raw data phenogram, 5VDE is placed between the coastal clade and the Shasta and Sierra clades, while in the null-corrected data, 5VDE is placed between the Shasta and Sierra clades (Fig. 3). Together, these phenograms indicate the atypical populations from Shasta County and the central Sierra Nevada are more similar to the congener *V. deliciosum* than the coastal populations of *V. parvifolium*.

While the phenograms represent the similarities among population averages, the Principle Coordinate Analysis (PCoA) maximizes differences among individuals. The PCoA based on the raw genotypes clustered samples by geographic collection and morphology on the first two axes, which explained 61% of the variation among individuals (Fig. 4). Four putative genetic clusters were indicated by the PCoA: *V. parvifolium* of typical morphology from the coastal areas, *Vaccinium* from the Sierra Nevada Mountains, *Vaccinium* from Shasta County, and the congener *V. deliciosum*. The first axis distinguishes the typical *V. parvifolium* from the Coastal Ranges and western Oregon from the collections of atypical *Vaccinium* from Shasta County and the Sierra Nevada. The single population of *V. deliciosum* is intermediate to these, but more similar to the atypical *Vaccinium* collections (Fig. 4). The second axis further separates the atypical *Vaccinium* collections by geographic region.

The admixture analyses using Structure estimated the most likely number of genetic clusters in the *Vaccinium* data set to be five ($dK_5 \approx 1$, all other $dK \approx 0$). Individual assignment to each cluster roughly followed population categories per Table 1. The populations of *V. parvifolium* of typical morphology were genetically similar and potentially admixed, being assigned to two clusters in varying proportions (orange and red). The five populations of atypical morphology from Shasta County were assigned to a single cluster (blue). The atypical populations *Vaccinium* from the Sierra Nevada were assigned to another cluster (purple), with some admixed with the blue cluster. The single population of *V. deliciosum* (5VDE) was distinct and assigned to a unique genetic cluster (green) (Fig. 5). These individual assignments are concordant with the PCoA results.

The Neighbor-Joining phenogram of anonymous genetic clusters identified in the Structure analysis was concordant with the PCoA and population phenograms. The cluster containing atypical Shasta County samples (blue) was more similar to the cluster containing atypical central Sierra Nevada collections (purple) and *V. deliciosum* (green) than to the clusters containing samples of typical morphologies (orange and red) (Fig. 5). These results are likely not an artifact of the null alleles as nulls were accounted for in the Structure analyses. Together, these results indicated the genetic differences among populations corresponded to geographic and morphological factors.

Discussion

Genetic Differentiation among Sampled Populations

The primary goal of this study was to establish whether populations of *Vaccinium* from Shasta County and the Sierra Nevada displaying atypical berry color and morphology were genetically similar to typical *V. parvifolium* from northwestern California and western Oregon and Washington. Analyses of five microsatellite

TABLE 3. ESTIMATES OF NEI'S (1972) GENETIC DISTANCE AMONG 20 POPULATIONS OF *VACCINIUM* SPP. Abbreviations follow Table 1. Values below diagonal are from raw data; values above diagonal from null-corrected allele frequencies.

|	1BH	1FLM	1LB	1SQC	1ULB	2ACF	2HC	2HD	2JS	2LD	2LR	2SFM	2SR	3I	3MP	4BC	4CC	4GH	4MC	5VDE
1BH		0.069	0.083	0.156	0.094	0.833	0.949	0.720	0.951	0.888	1.347	0.901	0.913	2.071	0.922	0.384	0.431	0.536	0.446	0.635
1FLM	0.060		0.065	0.154	0.081	0.885	0.879	0.726	0.916	1.036	1.425	0.881	0.813	2.764	0.922	0.315	0.401	0.499	0.387	0.655
1LB	0.083	0.051		0.110	0.016	0.797	0.913	0.753	0.802	1.076	1.496	0.931	0.837	2.158	0.906	0.287	0.447	0.468	0.366	0.675
1SQC	0.156	0.128	0.110		0.077	0.919	1.313	0.941	0.937	1.453	1.918	1.326	1.051	2.518	1.074	0.539	0.580	0.670	0.567	0.666
1ULB	0.094	0.059	0.016	0.077		0.795	1.033	0.766	0.821	1.190	1.515	0.999	0.882	2.365	0.951	0.401	0.502	0.611	0.496	0.666
2ACF	0.771	0.784	0.744	0.883	0.749		0.205	0.281	0.083	0.413	0.438	0.176	0.125	0.762	0.109	1.140	1.404	1.524	1.217	1.500
2HC	0.881	0.830	0.846	1.231	0.961	0.186		0.305	0.191	0.305	0.436	0.137	0.114	0.859	0.111	0.841	1.045	0.933	0.893	2.477
2HD	0.720	0.705	0.753	0.941	0.766	0.263	0.305		0.314	0.318	0.508	0.349	0.289	1.235	0.269	1.025	0.998	1.566	1.291	2.408
2JS	0.946	0.887	0.792	0.928	0.816	0.091	0.189	0.314		0.438	0.412	0.197	0.095	0.643	0.086	1.264	1.373	1.782	1.293	1.399
2LD	0.887	1.058	1.076	1.453	1.190	0.388	0.308	0.318	0.443		0.526	0.321	0.340	1.030	0.291	1.267	1.109	1.585	1.289	2.339
2LR	1.347	1.449	1.496	1.918	1.515	0.423	0.434	0.508	0.416	0.526		0.366	0.357	1.154	0.382	1.861	1.460	1.964	1.804	1.262
2SFM	0.901	0.904	0.931	1.326	0.999	0.152	0.139	0.349	0.199	0.321	0.366		0.102	0.797	0.112	0.962	1.135	1.556	1.168	1.646
2SR	0.906	0.803	0.825	1.037	0.869	0.108	0.102	0.293	0.091	0.344	0.359	0.102		0.668	0.065	1.033	1.165	1.515	1.163	1.794
3I	2.070	2.776	2.157	2.518	2.365	0.780	0.848	1.235	0.628	1.030	1.154	0.797	0.658		0.648	3.059	5.569	2.919	3.402	3.123
3MP	0.909	0.901	0.889	1.055	0.936	0.104	0.101	0.268	0.092	0.289	0.382	0.107	0.067	0.637		1.077	1.188	1.581	1.189	1.792
4BC	0.384	0.333	0.287	0.539	0.401	1.053	0.898	1.025	1.263	1.266	1.861	0.962	1.025	3.058	1.052		0.504	0.200	0.139	1.008
4CC	0.429	0.428	0.450	0.582	0.505	1.305	1.081	0.981	1.368	1.089	1.403	1.131	1.188	5.393	1.195	0.512		0.600	0.500	0.932
4GH	0.466	0.442	0.405	0.620	0.541	1.327	1.154	1.334	1.553	1.352	1.780	1.331	1.297	2.651	1.351	0.164	0.529		0.079	0.929
4MC	0.470	0.424	0.383	0.596	0.519	1.070	0.756	1.192	1.209	1.191	1.643	1.052	1.046	2.840	1.075	0.148	0.516	0.057		0.923
5VDE	0.615	0.651	0.650	0.634	0.635	1.390	2.214	2.301	1.363	2.349	1.268	1.648	1.742	2.989	1.736	1.016	0.932	0.975	0.970	
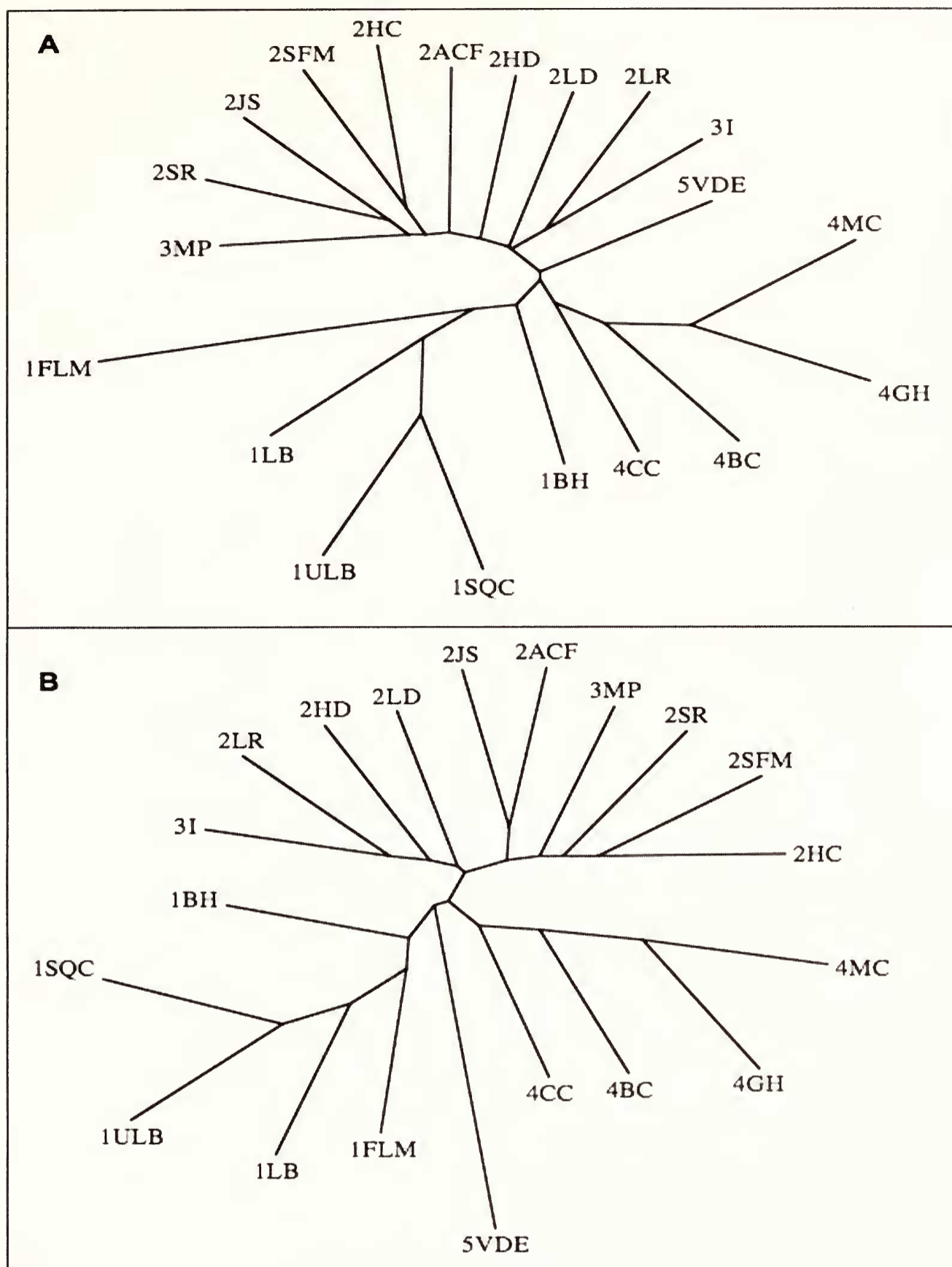
FIG. 3.   Unrooted Neighbor-Joining consensus population phenograms from Nei's (1972) genetic distances among 20 populations of *Vaccinium*. (A) The phenogram built from the raw data. (B) The phenogram built from null-corrected allele frequency data. Low statistical support was found for the topologies, with all branches occurring in <60% of bootstrap replicates.

loci revealed significant structure and differentiation along geographic and morphological groups. Four statistical analyses provide congruent evidence that the atypical populations of *Vaccinium* are genetically distinct from the *V. parvifolium* located in the Pacific coastal areas, and are more similar genetically to *V. deliciosum*, indicating additional systematic consideration may be warranted.

The level of differentiation among regions in this study was greater than the among-population variation reported for other *Vaccinium* species. The level of differentiation observed among populations within geographic regions in this study (10%) is consistent with the other *Vaccinium* reports (Yakimowski and Eckert 2008; Bell et al. 2009; Debnath 2009). Levels of differentiation among geographic regions was much higher (39%), indicating gene flow is restricted between the sampling categories (Table 1).

Varying levels of vegetative reproduction were identified across the collection. *Vaccinium* species
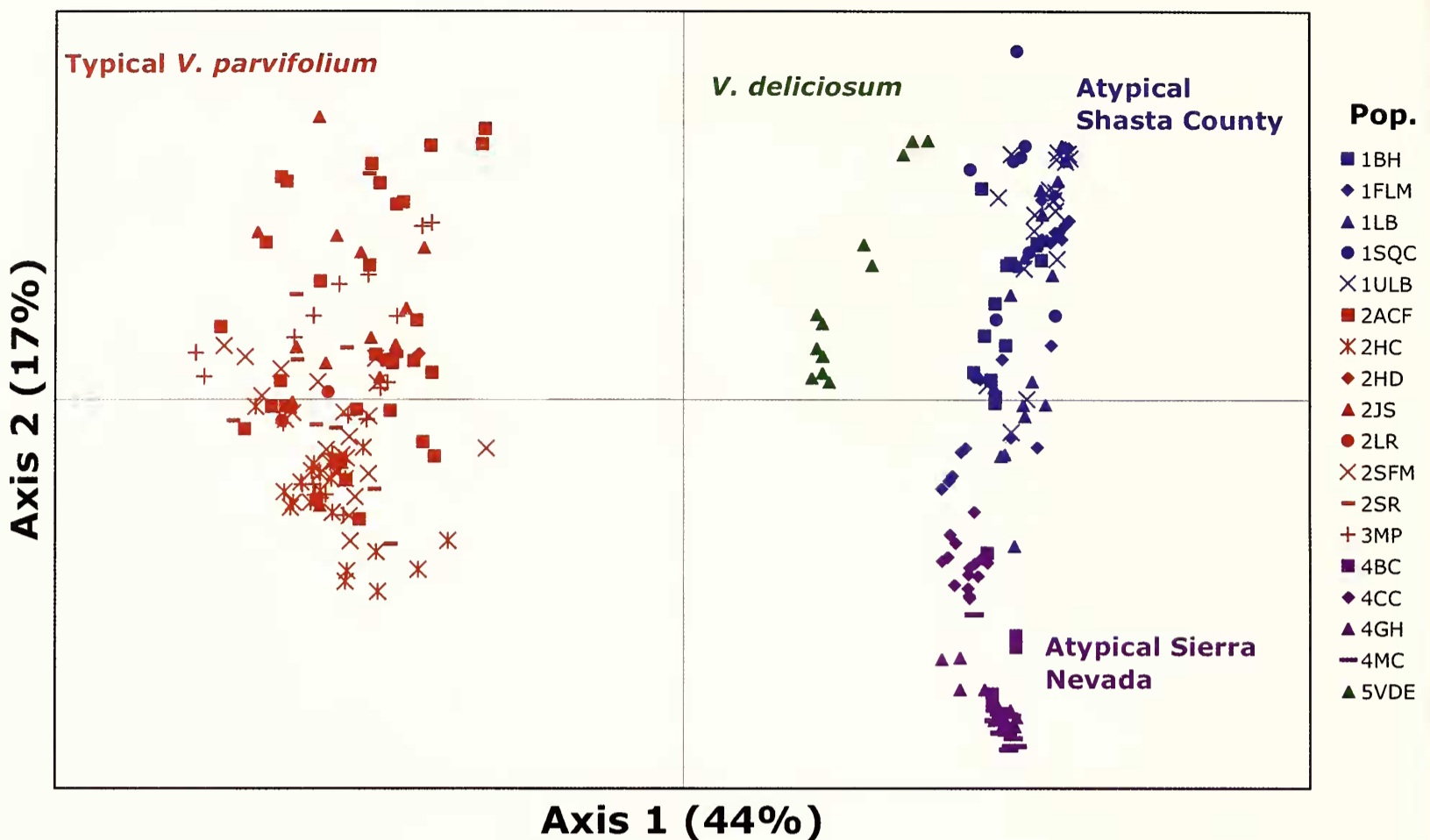
FIG. 4. Principal coordinate analysis reveals genetic structure among populations of *Vaccinium*. Each point represents one genet, and symbols correspond to population. Color relates to morphological variation depicted in Fig. 1.

are widely reported to reproduce clonally (Kreher et al. 2000; Yakimowski and Eckert 2008; Bell et al. 2009). *Vaccinium parvifolium* is reported to typically reproduce sexually but capable of producing sprouts in response to disturbance or damage (Wender et al. 2004) and in some cases via rhizomes (Gehrung 2001). Here, most populations displayed high levels of genet diversity (>0.8), but the collections from the Sierra Nevada displayed greater vegetative reproduction than those from the Coastal areas as indicated by smaller G/N ratios. Rhizomatous species may share resources among stems via a complex root system, providing a mechanism of survival in suboptimal or heterogeneous habitats (Wender et al. 2004). Thus, differences in vegetative reproduction in *Vaccinium* in different geographic regions may reflect an adaptive response to habitat quality or disturbance.

The presence of null alleles at four of the five loci may inflate measures of fixation and bias estimates of population differentiation, yet the low frequency of most nulls and their uneven distribution among populations minimized the impact on data interpretation. Null alleles are expected to be higher in frequency when primers designed for one species are used to amplify microsatellite loci in a related species, as was done in this study. However, if the lack of amplification resulted from fixed differences between *V. parvifolium* and the species for which the primers were designed (*V. corymbosum*,

highbush blueberry, Boches et al. 2005), we would expect null alleles to occur at high frequency in the majority of populations. Three lines of evidence indicate the impact of the null alleles was minimal and the genetic patterns observed were robust. First, as the AMOVA conducted for both raw data and that adjusted for null alleles produced congruent values, the results are likely conservative estimates of differentiation among populations and regions. Second, the Neighbor-Joining trees built with both raw data and null-corrected allele frequency data showed highly concordant topologies. Third, the Structure admixture analysis and individual assignment tests were parameterized to account for low frequencies of null alleles (Falush et al. 2007) and identified genetic structure concordant to those resolved with other analyses.

A high number of unique alleles were detected in various *Vaccinium* populations. A total of 27 alleles (26%) were only observed in a single population, with 12 of the 20 populations displaying at least one unique allele (Supplemental Material). Private alleles were more frequent in the typical coastal and atypical Sierra Nevada populations than the atypical populations of Shasta County, indicating gene flow is restricted among regions. A relatively small number of migrants per generation ($N_e m = 4$) is sufficient to maintain genetic similarity between demes (Hartl and Clark 2007). Restricted gene flow between the Sierra Nevada and Coast Ranges seems
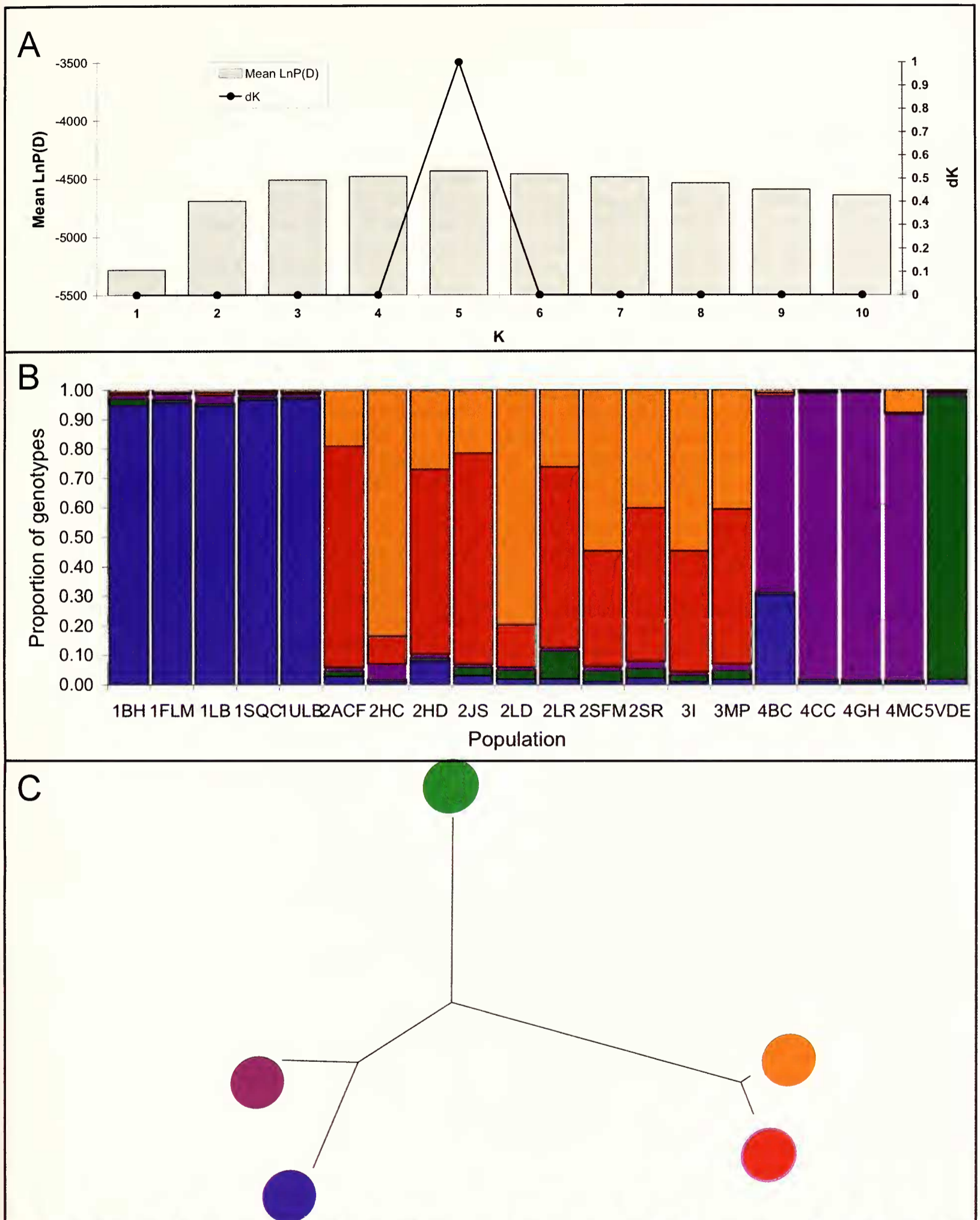
FIG. 5.   Patterns of genetic differentiation revealed by admixture analyses. A) The average likelihood of analyses identified five genetic clusters (dK) in the collection. B) Assignment of individuals to the five genetic clusters. Each vertical bar represents one sampled population. Color corresponds to each genetic cluster; bars composed of multiple colors represent potentially admixed populations. Refer to Table 1 for sample sizes, which vary among populations. C) Neighbor-Joining phenogram of the genetic distances between the five genetic clusters. Colors match genetic clusters from (B).

reasonable given the geographic distance between regions. A lack of gene flow between the Coast Ranges and Shasta County may be more surprising. Some populations of differing morphology are closer geographically (e.g., 1SQC and 2SFM separated by 77 km) than are two populations of the typical phenotype (e.g., 1SQC and 3MP separated by 425 km). Yet the distant populations of similar morphology are significantly more similar genetically. The lack of private alleles in the Shasta County populations may be due to a number of demographic factors requiring additional study (e.g., inbreeding). The locus CA23F displayed fixed differences between geographic regions, with samples from the coastal areas being homozygous for a smaller allele (164 bp) and all other samples from the Sierra Nevada being homozygous for a larger allele (167 bp). Given the expected high rate of mutation at SSR loci, the lack of variation at this locus may indicate it is linked to a functional variant under strong selection. Additional study will be required to determine if fixed allelic differences correspond to any adaptive variation.

Systematic Interpretations of Differentiation

The second objective of this study was to determine whether any genetic differences between populations might be indicative of greater systematic divergence than currently described in the taxonomic treatments. Morphologically, *V. parvifolium* forms a distinct clade separate from the *V. deliciosum/ovalifolium* complex (Vander Kloet and Dickinson 1999). *Vaccinium parvifolium* is typically larger than related *Vaccinium* species, and is characterized by slower germination and apical meristem development in seedlings, and persistent juvenile leaf morphology, distinctive biochemical properties and a taproot system unique in the section. Phenotypic variation within *V. parvifolium* is well noted (Vander Kloet and Dickinson 1999; Gehrung 2001). Gehrung (2001) noted the rhizomatous growth and distinct berry color in the Sierra Nevada populations, and suggested elevation to subspecific status may be appropriate upon further study. However, if variation is due to phenotypic plasticity among panmictic, we would expect low genetic differentiation between morphologically distinct populations, which is not the pattern observed here. Further, phenotypic variation in the related *V. membranaceum* Douglas ex Torr.was shown to be environmentally induced (Schultz 1944 in Vander Kloet and Dickinson 1999); common garden experiments are required to determine the genetic basis of morphological variation in *V. parvifolium*. *Vaccinium deliciosum*, in contrast, is rhizomatous, forming dense stands of ramets, and displays very little phenotypic variation (Gerhung 2001). Genetic examinations have also separated these species into different clades of section *Myrtillus*. Cytological evidence reported *V. parvifolium* from the Pacific Coast (British Columbia, Canada and Washington state) as diploid ($n = 12$) while *V. deliciosum was* described as tetraploid ($n = 24$) (Vander Kloet and Dickinson 1999). Combined analyses of nuclear ribosomal ITS and chloroplast *matK* sequences clustered *V. parvifolium* with *V. ovalifolium* and indicated this clade to be directly ancestral the *V. deliciosum* clade (Powell and Kron 2002).

The genetic differentiation observed in this study indicates greater diversity is present in *V. parvifolium* than previously reported in Northern California. The Shasta County and Sierra Nevada populations are more similar genetically to the congener *V. deliciosum* than they are to the coastal populations. Possible taxonomic revisions may involve expanding the definition of *V. parvifolium* to allow variation in berry color and high levels of genetic differentiation. Alternatively, a novel *Vaccinium* species or subspecies may be necessary to accurately reflect the morphological and genetic variation. Given the divergence of the Shasta County and Sierra Nevada populations from the typical *V. parvifolium*, the latter option may be more accurate. Additional morphological and ecologic study of these populations is warranted.

Ecological and Conservation Implications

Given the important role of *Vaccinium* shrubs in coniferous forests (Vila et al. 2004; Wender et al. 2004; Hanley 2005), an accurate understanding of the species diversity and distribution is necessary to understand the ecological role of this genus. The genetic differentiation observed among populations of *Vaccinium* in northern California may reflect functional or adaptive divergence among geographic regions, in particular with regard to fruit color. Avian preference of berry color may vary temporally and with background foliage color (Burns and Dalen 2002; Honkavaara et al. 2004). In *Rubus spectabilis* Pursh, seed germination of different color morphs varies with soil type, indicating fruit color may correlate with adaptive differentiation (Traveset and Willson 1998). In addition to berry color, differences in the extent of clonal growth patterns can affect the functional structure of populations. Clonal growth forms may allow genets to survive over a much longer timespan than individual ramets, and can significantly decrease effective population size (Persson and Gustavsson 2001). Observational and manipulative studies will be required to confirm if these differences constitute ecologically significant variation.

While *V. parvifolium* is not a species of conservation concern, management plans should account for the distinct gene pools identified in

these *Vaccinium* collections. The distinct morphotype from Shasta County is of interest to USDA Forest Service management plans as it is consistent with growing evidence of a distinctive biodiversity in the Shasta Lake region, particularly if distinct subspecies or cryptic species are designated. Given the significant genetic differentiation among geographic groups (coastal, Shasta County, and Sierra Nevada), artificial movement of germplasm should be restricted to within and not among regions. These observations are interesting as the *Vaccinium* berries are fleshy and palatable, and provide forage for numerous species in coniferous forests (Wender et al. 2004), though the dispersal ranges are likely small relative to the habitat range described for *V. parvifolium* (Rosatti 2003).

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